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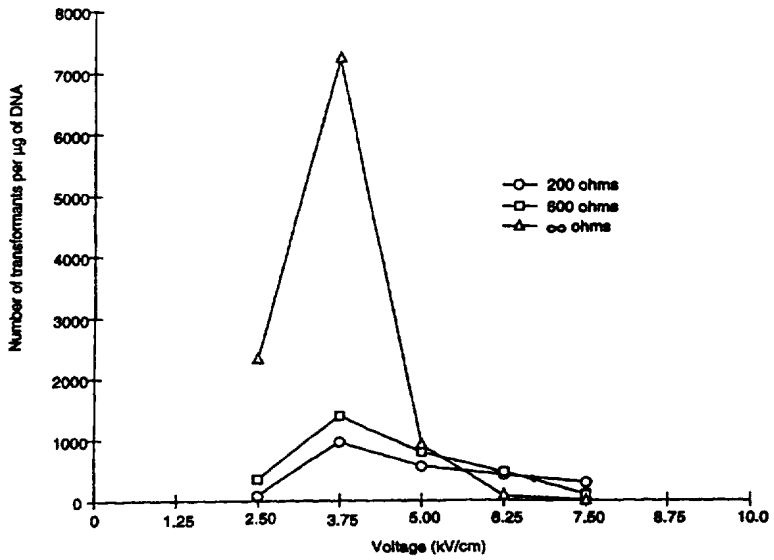

(57) Abstract

Methods for transforming *Pichia methanolica*, and DNA molecules useful in transformation of *P. methanolica*, are disclosed. *P. methanolica* cells are exposed, in the presence of DNA molecules, to a pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm and a pulse duration of from 1 to 40 milliseconds, whereby the DNA molecules are introduced into the cells. The DNA molecules may comprise an expression unit that includes a transcription promoter of a *P. methanolica* gene operably linked to a segment encoding a polypeptide or protein of interest. The DNA molecules may also encode a selectable marker, such as a *P. methanolica* ADE2 gene. Cells transformed according to the invention may be used in production systems for the preparation of proteins of commercial importance.

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Description

TRANSFORMATION OF *PICHIA METHANOLICA*

Background of the Invention

Methylotrophic yeasts are those yeasts that are able to utilize methanol as a sole source of carbon and energy. Species of yeasts that have the biochemical pathways necessary for methanol utilization are classified in four genera, Hansenula, Pichia, Candida, and Torulopsis. These genera are somewhat artificial, having been based on cell morphology and growth characteristics, and do not reflect close genetic relationships (Billon-Grand, Mycotaxon 35:201-204, 1989; Kurtzman, Mycologia 84:72-76, 1992). Furthermore, not all species within these genera are capable of utilizing methanol as a source of carbon and energy. As a consequence of this classification, there are great differences in physiology and metabolism between individual species of a genus.

Methylotrophic yeasts are attractive candidates for use in recombinant protein production systems. Some methylotrophic yeasts have been shown to grow rapidly to high biomass on minimal defined media. Certain genes of methylotrophic yeasts are tightly regulated and highly expressed under induced or de-repressed conditions, suggesting that promoters of these genes might be useful for producing polypeptides of commercial value. See, for example, Faber et al., Yeast 11:1331, 1995; Romanos et al., Yeast 8:423, 1992; and Cregg et al., Bio/Technology 11:905, 1993.

Development of methylotrophic yeasts as hosts for use in recombinant production systems has been slow, due in part to a lack of suitable materials (e.g., promoters, selectable markers, and mutant host cells) and methods (e.g., transformation techniques). The most highly developed methylotrophic host systems utilize *Pichia pastoris* and *Hansenula polymorpha* (Faber et al., Curr. Genet. 25:305-310, 1994; Cregg et al., ibid.; Romanos et al., ibid.; U.S. Patent No. 4,855,242; U.S. Patent No. 4,857,467; U.S. Patent No. 4,879,231; and U.S. Patent No. 4,929,555).

There remains a need in the art for methods of transforming additional species of methylotrophic yeasts and for using the transformed cells to produce polypeptides of economic importance, including industrial enzymes and pharmaceutical proteins. The present invention provides such methods as well as other, related advantages.

Summary of the Invention

The present invention provides methods for introducing DNA molecules into *Pichia methanolica* cells and cells transformed according to these methods.

Within one aspect of the invention, the methods comprise exposing a *Pichia methanolica* cell, in the presence of a linear DNA molecule, to an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm and a time constant of from 1 to 40 milliseconds, whereby the DNA molecule is introduced into the cell. Within one embodiment of the invention, the DNA molecule comprises a segment encoding a polypeptide, other than a *P. methanolica* polypeptide, operably linked to a *P. methanolica* gene transcription promoter and a *P. methanolica* gene transcription terminator. Within a preferred embodiment, the transcription promoter is a *P. methanolica AUG1* gene promoter, which, within one embodiment, comprises a sequence of nucleotides as shown in SEQ ID NO:2 from nucleotide 24 to nucleotide 1354. The DNA molecule may further comprise a selectable marker gene that complements a mutation in the host cell. Within one embodiment, the selectable marker gene is a *P. methanolica* gene, such as a *P. methanolica ADE2* gene.

Within a second aspect of the invention there are provided methods for transforming *P. methanolica* with heterologous DNA, comprising exposing a population of *P. methanolica* cells, in the presence of heterologous linear DNA molecules, to an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm and a time constant of from 1 to 40 milliseconds, whereby the heterologous DNA is introduced into at least a portion of the population, and recovering cells into which the DNA has been introduced. Within one embodiment of the invention, from 10^3 to 10^5 cells are recovered per microgram of heterologous DNA. Within another embodiment, from 0.9×10^4 to 1.1×10^4 cells are recovered per microgram of heterologous DNA. Within a further embodiment, the methods further comprise the step of recovering integrative transformants from the recovered cells, such as by culturing the cells in a growth medium comprising sorbitol as a carbon source. Within an additional embodiment, the population of cells is in early log phase growth.

Within other aspects, the present invention provides *Pichia methanolica* cells transformed by the methods disclosed above.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawings.

Brief Description of the Drawings

Fig. 1 illustrates the effects of field strength and pulse duration on electroporation efficiency of *P. methanolica*.

Fig. 2 is a schematic diagram of a recombination event between plasmid pCZR140 and *P. methanolica* genomic DNA.

Fig. 3 is a schematic diagram of a recombination event between plasmid pCZR137 and *P. methanolica* genomic DNA.

Detailed Description of the Invention

Prior to setting forth the invention in more detail, it will be useful to define certain terms used herein:

Early log phase growth--That phase of cellular growth in culture when the cell concentration is from 2 x 10^6 cells/ml to 8 x 10^6 cells/ml.

Heterologous DNA--A DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species so long as that host DNA is combined with non-host DNA. For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule.

Integrative transformants--Cells into which have been introduced heterologous DNA, wherein the heterologous DNA has become integrated into the genomic DNA of the cells.

Linear DNA--DNA molecules having free 5' and 3' ends, that is non-circular DNA molecules. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

Operably linked--The term operably linked indicates that DNA segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

As noted above, the present invention provides methods for introducing DNA into cells of the methylotrophic yeast *Pichia methanolica*, and for selecting cells into which the DNA has been introduced. Those skilled in the art will recognize that transformation of cells with both homologous DNA (DNA from the host species) and heterologous DNA is a prerequisite to a large number of diverse biological applications. The methods of the present invention are particularly well suited to the preparation of cells transformed with heterologous DNA, which cells can be used for the production of polypeptides and proteins, including polypeptides and proteins of higher organisms, including humans. The present invention further provides for the transformation of *Pichia methanolica* cells with other DNA molecules, including DNA libraries and synthetic DNA molecules. The invention thus provides techniques that can be used to express genetically diverse libraries to produce products that are screened for novel biological activities, to engineer cells for use as targets for the screening of compound libraries, and to genetically modify cells to enhance their utility within other processes.

Strains of *Pichia methanolica* are available from the American Type Culture Collection (Rockville, MD) and other repositories. Within one embodiment of the invention, cells to be transformed with heterologous DNA will have a mutation that can be complemented by a gene (a "selectable marker") on the heterologous DNA molecule. This selectable marker allows the transformed cells to grow under conditions in which untransformed cells cannot multiply ("selective conditions"). The general principles of selection are well known in the art. Commonly used selectable markers are genes that encode enzymes required for the synthesis of amino acids or nucleotides. Cells having mutations in these genes cannot grow in media lacking

the specific amino acid or nucleotide unless the mutation is complemented by the selectable marker. Use of such "selective" culture media ensures the stable maintenance of the heterologous DNA within the host cell. A preferred selectable marker of this type for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21). The *ADE2* gene, when transformed into an *ade2* host cell, allows the cell to grow in the absence of adenine. The coding strand of a representative *P. methanolica ADE2* gene sequence is shown in SEQ ID NO:1. The sequence illustrated includes 1006 nucleotides of 5' non-coding sequence and 442 nucleotides of 3' non-coding sequence, with the initiation ATG codon at nucleotides 1007-1009. Within a preferred embodiment of the invention, a DNA segment comprising nucleotides 407-2851 is used as a selectable marker, although longer or shorter segments could be used as long as the coding portion is operably linked to promoter and terminator sequences. Those skilled in the art will recognize that this and other sequences provided herein represent single alleles of the respective genes, and that allelic variation is expected to exist. Any functional *ADE2* allele can be used within the present invention. Other nutritional markers that can be used within the present invention include the *P. methanolica ADE1*, *HIS3*, and *LEU2* genes, which allow for selection in the absence of adenine, histidine, and leucine, respectively. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Gene-deficient mutants can be prepared by known methods, such as site-directed mutagenesis. *P. methanolica* genes can be cloned on the basis of homology with their counterpart *Saccharomyces cerevisiae* genes. The *ADE2* gene disclosed herein was given its designation on the basis of such homology.

Within another embodiment of the invention, a dominant selectable marker is used, thereby obviating the need for mutant host cells. Dominant selectable markers are those that are able to provide a growth advantage to wild-type cells. Typical dominant selectable markers are genes that provide resistance to antibiotics, such as neomycin-type antibiotics (e.g., G418), hygromycin B, and bleomycin/phleomycin-type antibiotics (e.g., Zeocin™; available from Invitrogen Corporation, San Diego, CA). A preferred dominant selectable marker for use in *P. methanolica* is the *Sh bla* gene, which inhibits the activity of Zeocin™ .

Electroporation is used within the present invention to facilitate the introduction of DNA into *P. methanolica* cells. Electroporation is the process of using a pulsed electric field to transiently permeabilize cell membranes, allowing macromolecules, such as DNA, to pass into cells. Electroporation has been described for use with mammalian (e.g., Neumann et al., EMBO J. 1:841-845, 1982) and fungal (e.g., Meilhoc et al., Bio/Technology 8:223-227, 1990) host cells. However, the actual mechanism by which DNA is transferred into the cells is not well understood. For transformation of *P. methanolica*, it has been found that electroporation is surprisingly efficient when the cells are exposed to an exponentially decaying, pulsed electric

field having a field strength of from 2.5 to 4.5 kV/cm and a time constant (τ) of from 1 to 40 milliseconds. The time constant τ is defined as the time required for the initial peak voltage V_0 to drop to a value of V_0/e. The time constant can be calculated as the product of the total resistance and capacitance of the pulse circuit, i.e., $\tau = R \times C$. Typically, resistance and capacitance are either preset or may be selected by the user, depending on the electroporation equipment selected. In any event, the equipment is configured in accordance with the manufacturer's instructions to provide field strength and decay parameters as disclosed above. Electroporation equipment is available from commercial suppliers (e.g., BioRad Laboratories, Hercules, CA).

DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide or protein production, the DNA molecules will include, in addition to the selectable marker disclosed above, an expression casette comprising a transcription promoter, a DNA segment (e.g., a cDNA) encoding the polypeptide or protein of interest, and a transcription terminator. These elements are operably linked to provide for transcription of the DNA segment of interest. It is preferred that the promoter and terminator be that of a *P. methanolica* gene. A preferred promoter is that of a *P. methanolica* alcohol utilization gene (*AUG1*), a representative coding strand sequence of which is shown in SEQ ID NO:2. Within SEQ ID NO:2, the initiation ATG codon is at nucleotides 1355-1357. Nucleotides 1-23 of SEQ ID NO:2 are non-*AUG1* polylinker sequence. It is particularly preferred to utilize as a promoter a segment comprising nucleotides 24-1354 of SEQ ID NO:2, although additional upstream sequence can be included. *P. methanolica* contains a second alcohol utilization gene, *AUG2*, the promoter of which can be used within the present invention. Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. The DNA molecules will further include a selectable marker to allow for identification, selection, and maintenance of transformants. The DNA molecules may further contain additional elements, such an origin of replication and a selectable marker that allow amplification and maintenance of the DNA in an alternate host (e.g., *E. coli*). To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment, comprising the promoter--gene of interest--terminator plus selectable marker, flanked at both ends by host DNA sequences. This is conveniently accomplished by including 3' untranslated DNA sequence at the downstream end of the expression segment and relying on the promoter sequence at the 5' end. When using linear DNA, the expression segment will be flanked by cleavage sites to allow for linearization of the molecule and separation of the expression segment from other sequences (e.g., a bacterial origin of replication and selectable marker). Preferred such cleavage sites are those that are recognized by restriction endonucleases that cut infrequently within a DNA sequence, such as those that recognize 8-base target sequences (e.g., Not I).

Proteins that can be produced in *P. methanolica* using the methods of the present invention include proteins of industrial and pharmaceutical interest. Such proteins include enzymes such as lipases, cellulases, and proteases; enzyme inhibitors, including protease inhibitors; growth factors such as platelet derived growth factor, fibroblast growth factors, and epidermal growth factor; cytokines such as erythropoietin and thrombopoietin; and hormones such as insulin, leptin, and glucagon .

For use within the present invention, *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred culture medium is YEPD (Table 1). The cells may be passaged by dilution into fresh culture medium or stored for short periods on plates under refrigeration. For long-term storage, the cells are preferably kept in a 50% glycerol solution at -70°C.

Table 1

YEPD

2% D-glucose
2% Bacto™ Peptone (Difco Laboratories, Detroit, MI)
1% Bacto™ yeast extract (Difco Laboratories)
0.004% adenine
0.006% L-leucine

ADE D

0.056% -Ade -Trp -Thr powder
0.67% yeast nitrogen base without amino acids
2% D-glucose
0.5% 200X tryptophan, threonine solution

ADE DS

0.056% -Ade -Trp -Thr powder
0.67% yeast nitrogen base without amino acids
2% D-glucose
0.5% 200X tryptophan, threonine solution
18.22% D-sorbitol

LEU D

0.052% -Leu -Trp -Thr powder
0.67% yeast nitrogen base without amino acids
2% D-glucose
0.5% 200X tryptophan, threonine solution

HIS D

0.052% -His -Trp -Thr powder
0.67% yeast nitrogen base without amino acids

2% D-glucose

0.5% 200X tryptophan, threonine solution

URA D

0.056% -Ura -Trp -Thr powder

0.67% yeast nitrogen base without amino acids

2% D-glucose

0.5% 200X tryptophan, threonine solution

Table 1, continued

URA DS

0.056% -Ura -Trp -Thr powder

0.67% yeast nitrogen base without amino acids

2% D-glucose

0.5% 200X tryptophan, threonine solution

18.22% D-sorbitol

-Leu -Trp -Thr powder

powder made by combining 4.0 g adenine, 3.0 g arginine, 5.0 g aspartic acid, 2.0 g histidine, 6.0 g isoleucine, 4.0 g lysine, 2.0 g methionine, 6.0 g phenylalanine, 5.0 g serine, 5.0 g tyrosine, 4.0 g uracil, and 6.0 g valine (all L- amino acids)

-His -Trp -Thr powder

powder made by combining 4.0 g adenine, 3.0 g arginine, 5.0 g aspartic acid, 6.0 g isoleucine, 8.0 g leucine, 4.0 g lysine, 2.0 g methionine, 6.0 g phenylalanine, 5.0 g serine, 5.0 g tyrosine, 4.0 g uracil, and 6.0 g valine (all L- amino acids)

-Ura -Trp -Thr powder

powder made by combining 4.0 g adenine, 3.0 g arginine, 5.0 g aspartic acid, 2.0 g histidine, 6.0 g isoleucine, 8.0 g leucine, 4.0 g lysine, 2.0 g methionine, 6.0 g phenylalanine, 5.0 g serine, 5.0 g tyrosine, and 6.0 g valine (all L- amino acids)

-Ade -Trp -Thr powder

powder made by combining 3.0 g arginine, 5.0 g aspartic acid, 2.0 g histidine, 6.0 g isoleucine, 8.0 g leucine, 4.0 g lysine, 2.0 g methionine, 6.0 g phenylalanine, 5.0 g serine, 5.0 g tyrosine, 4.0 g uracil, and 6.0 g valine (all L- amino acids)

200X tryptophan, threonine solution

3.0% L-threonine, 0.8% L-tryptophan in H_2O

For plates, add 1.8% Bacto™ agar (Difco Laboratories)

Electroporation of *P. methanolica* is preferably carried out on cells in early log phase growth. Cells are streaked to single colonies on solid media, preferably solid YEPD. After about 2 days of growth at 30°C, single colonies from a fresh plate are used to inoculate the desired volume of rich culture media (e.g., YEPD) to a cell density of about 5 - 10 x 10^5 cells/ml. Cells are incubated at about 25 - 35°C, preferably 30°C, with vigorous shaking, until they are in early log phase. The cells are then harvested, such as by centrifugation at 3000 x g for 2-3 minutes, and resuspended. Cells are made electrocompetent by reducing disulfide bonds in the cell walls, equilibrating them in an ionic solution that is compatible with the electroporation conditions, and chilling them. Cells are typically made electrocompetent by incubating them in a buffered solution at pH 6-8 containing a reducing agent, such as dithiothreitol (DTT) or β-mercaptoethanol (BME), to reduce cell wall proteins to facilitate subsequent uptake of DNA. A preferred incubation buffer in this regard is a fresh solution of 50

mM potassium phosphate buffer, pH 7.5, containing 25 mM DTT. The cells are incubated in this buffer (typically using one-fifth the original culture volume) at about 30°C for about 5 to 30 minutes, preferably about 15 minutes. The cells are then harvested and washed in a suitable electroporation buffer, which is used ice-cold. Suitable buffers in this regard include pH 6-8 solutions containing a weak buffer, divalent cations (e.g., Mg^{++}, Ca^{++}) and an osmotic stabilizer (e.g., a sugar). After washing, the cells are resuspended in a small volume of the buffer, at which time they are electrocompetent and can be used directly or aliquotted and stored frozen (preferably at -70°C). A preferred electroporation buffer is STM (270 mM sucrose, 10 mM Tris, pH 7.5, 1 mM $MgCl_2$). Within a preferred protocol, the cells are subjected to two washes, first in the original culture volume of ice-cold buffer, then in one-half the original volume. Following the second wash, the cells are harvested and resuspended, typically using about 3-5 ml of buffer for an original culture volume of 200 ml.

Electroporation is carried out using a small volume of electrocompetent cells (typically about 100 µl) and up to one-tenth volume of linear DNA molecules. For example, 0.1 ml of cell suspension in a buffer not exceeding 50 mM in ionic strength is combined with 0.1-10 µg of DNA (vol. ≤ 10 µl). This mixture is placed in an ice-cold electroporation cuvette and subjected to a pulsed electric field of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant of from 1 to 40 milliseconds, preferably 10-30 milliseconds, more preferably 15-25 milliseconds, most preferably about 20 milliseconds, with exponential decay. The actual equipment settings used to achieve the desired pulse parameters will be determined by the equipment used. When using a BioRad (Hercules, CA) Gene Pulser™ electroporator with a 2 mm electroporation cuvette, resistance is set at 600 ohms or greater, preferably "infinite" resistance, and capacitance is set at 25 µF to obtain the desired field characteristics. After being pulsed, the cells are diluted approximately 10X into 1 ml of YEPD broth and incubated at 30°C for one hour.

The cells are then harvested and plated on selective media. Within a preferred embodiment, the cells are washed once with a small volume (equal to the diluted volume of the electroporated cells) of 1X yeast nitrogen base (6.7 g/L yeast nitrogen base without amino acids; Difco Laboratories, Detroit, MI), and plated on minimal selective media. Cells having an *ade2* mutation that have been transformed with an *ADE2* selectable marker can be plated on a minimal medium that lacks adenine, such as ADE D (Table 1) or ADE DS (Table 1). In a typical procedure, 250 µl aliqouts of cells are plated on 4 separate ADE D or ADE DS plates to select for Ade^+ cells.

P. methanolica recognizes certain infrequently occuring sequences, termed autonomously replicating sequences (ARS), as origins of DNA replication, and these sequences may fortuitously occur within a DNA molecule used for transformation, allowing the transforming DNA to be maintained extrachromosomally. However, integrative transformants are generally preferred for use in protein production systems. Integrative transformants have a profound growth advantage over ARS transformants on selective media containing sorbitol as a

carbon source, thereby providing a method for selecting integrative transformants from among a population of transformed cells. ARS sequences have been found to exist in the *ADE2* gene and, possibly, the *AUG1* gene of *P. methanolica*. *ade2* host cells of *Pichia methanolica* transformed with an *ADE2* gene can thus become Ade^+ by at least two different modes. The ARS within the *ADE2* gene allows unstable extrachromosomal maintenance of the transforming DNA (Hiep et al., Yeast 9:1189-1197, 1993). Colonies of such transformants are characterized by slower growth rates and pink color due to prolific generation of progeny that are Ade^-. Transforming DNA can also integrate into the host genome, giving rise to stable transformants that grow rapidly, are white, and that fail to give rise to detectable numbers of Ade^- progeny. ADE D plates allow the most rapid growth of transformed cells, and unstable and stable transformants grow at roughly the same rates. After 3-5 days of incubation on ADE D plates at 30°C stable transformant colonies are white and roughly twice the size of unstable, pink transformants. ADE DS plates are more selective for stable transformants, which form large (≈5 mm) colonies in 5-7 days, while unstable (ARS-maintained) colonies are much smaller (≈1 mm). The more selective ADE DS media is therefore preferred for the identification and selection of stable transformants. For some applications, such as the screening of genetically diverse libraries for rare combinations of genetic elements, it is sometimes desirable to screen large numbers of unstable transformants, which have been observed to outnumber stable transformants by a factor of roughly 100. In such cases, those skilled in the art will recognize the utility of plating transformant cells on less selective media, such as ADE D.

Integrative transformants are preferred for use in protein production processes. Such cells can be propagated without continuous selective pressure because DNA is rarely lost from the genome. Integration of DNA into the host chromosome can be confirmed by Southern blot analysis. Briefly, transformed and untransformed host DNA is digested with restriction endonucleases, separated by electrophoresis, blotted to a support membrane, and probed with appropriate host DNA segments. Differences in the patterns of fragments seen in untransformed and transformed cells are indicative of integrative transformation. Restriction enzymes and probes can be selected to identify transforming DNA segments (e.g., promoter, terminator, heterologous DNA, and selectable marker sequences) from among the genomic fragments.

Differences in expression levels of heterologous proteins can result from such factors as the site of integration and copy number of the expression cassette and differences in promoter activity among individual isolates. It is therefore advantageous to screen a number of isolates for expression level prior to selecting a production strain. A variety of suitable screening methods are available. For example, transformant colonies are grown on plates that are overlayed with membranes (e.g., nitrocellulose) that bind protein. Proteins are released from the cells by secretion or following lysis, and bind to the membrane. Bound protein can then be assayed using known methods, including immunoassays. More accurate analysis of expression levels can be obtained by culturing cells in liquid media and analyzing conditioned media or cell lysates, as appropriate. Methods for concentrating and purifying proteins from media and lysates

will be determined in part by the protein of interest. Such methods are readily selected and practiced by the skilled practitioner.

For small-scale protein production (e.g., plate or shake flask production), *P. methanolica* transformants that carry an expression cassette comprising a methanol-regulated promoter (such as the *AUG1* promoter) are grown in the presence of methanol and the absence of interfering amounts of other carbon sources (e.g., glucose). For small-scale experiments, including preliminary screening of expression levels, transformants may be grown at 30°C on solid media containing, for example, 20 g/L Bacto-agar (Difco), 6.7 g/L yeast nitrogen base without amino acids (Difco), 10 g/L methanol, 0.4 μg/L biotin, and 0.56 g/L of -Ade -Thr -Trp powder. Because methanol is a volatile carbon source it is readily lost on prolonged incubation. A continuous supply of methanol can be provided by placing a solution of 50% methanol in water in the lids of inverted plates, whereby the methanol is transferred to the growing cells by evaporative transfer. In general, not more than 1 mL of methanol is used per 100-mm plate. Slightly larger scale experiments can be carried out using cultures grown in shake flasks. In a typical procedure, cells are cultivated for two days on minimal methanol plates as disclosed above at 30°C, then colonies are used to inoculate a small volume of minimal methanol media (6.7 g/L yeast nitrogen base without amino acids, 10 g/L methanol, 0.4 μg/L biotin) at a cell density of about 1 x 10^6 cells/ml. Cells are grown at 30°C. Cells growing on methanol have a high oxygen requirement, necessitating vigorous shaking during cultivation. Methanol is replenished daily (typically 1/100 volume of 50% methanol per day).

For production scale culturing, fresh cultures of high producer clones are prepared in shake flasks. The resulting cultures are then used to inoculate culture medium in a fermenter. Typically, a 500 ml culture in YEPD grown at 30°C for 1-2 days with vigorous agititation is used to inoculate a 5-liter fermenter. The cells are grown in a suitable medium containing salts, glucose, biotin, and trace elements at 28°C, pH 5.0, and >30% dissolved O_2. After the initial charge of glucose is consumed (as indicated by a decrease in oxygen consumption), a glucose/methanol feed is delivered into the vessel to induce production of the protein of interest. Because large-scale fermentation is carried out under conditions of limiting carbon, the presence of glucose in the feed does not repress the methanol-inducible promoter.

The invention is further illustrated by the following non-limiting examples.

Examples

Example 1

P. methanolica cells (strain CBS6515 from American Type Culture Collection, Rockville, MD) were mutagenized by UV exposure. A killing curve was first generated by plating cells onto several plates at approximately 200-250 cells/plate. The plates were then exposed to UV radiation using a G8T5 germicidal lamp (Sylvania) suspended 25 cm from the surfaces of the plates for periods of time as shown in Table 2. The plates were then protected from visible light sources and incubated at 30°C for two days.

Table 2

Viable Cells

Time	Plate 1	Plate 2	Average
0 sec.	225	229	227
1 sec.	200	247	223
2 sec.	176	185	181
4 sec.	149	86	118
8 sec.	20	7	14
16 sec.	0	2	1

Large-scale mutagenesis was then carried out using a 2-second UV exposure to provide about 20% killing. Cells were plated at approximately 10^4 cells/plate onto eight YEPD plates that were supplemented with 100 mg/L each of uracil, adenine, and leucine, which were added to supplement the growth of potential auxotrophs having the cognate deficiencies. Following UV exposure the plates were wrapped in foil and incubated overnight at 30°C. The following day the colonies on the plates (~10^5 total) were resuspended in water and washed once with water. An amount of cell suspension sufficient to give an OD_{600} of 0.1 - 0.2 was used to inoculate 500 ml of minimal broth made with yeast nitrogen base without amino acids or ammonia, supplemented with 1% glucose and 400 µg/L biotin. The culture was placed in a 2.8 L baffled Bell flask and shaken vigorously overnight at 30°C. The following day the cells had reached an OD_{600} of ~1.0 - 2.0. The cells were pelleted and resuspended in 500 ml of minimal broth supplemented with 5 g/L ammonium sulfate. The cell suspension was placed in a 2.8 L baffled Bell flask and shaken vigorously at 30°C for 6 hours. 50 ml of the culture was set aside in a 250-ml flask as a control, and to the remainder of the culture was added 1 mg nystatin to select for auxotrophic mutants (Snow, Nature 211:206-207, 1966). The cultures were incubated with shaking for an additional hour. The control and nystatin-treated cells were then harvested by centrifugation and washed with water three times. The washed cells were resuspended to an OD_{600} of 1.0 in 50% glycerol and frozen. Titering of nystatin-treated cells versus the control cells for colony forming units revealed that nystatin enrichment had decreased the number of viable cells by a factor of 10^4.

10^{-2} dilutions of nystatin-treated cells were plated on 15 YEPD plates. Colonies were replica-plated onto minimal plates (2% agar, 1 x YNB, 2% glucose, 400 µg/L biotin). The frequency of auxotrophs was about 2 - 4%. Approximately 180 auxotrophic colonies were picked to YEPD + Ade, Leu, Ura plates and replica-plated to various dropout plates. All of the auxotrophs were Ade^-. Of these, 30 were noticably pink on dropout plates (LEU D, HIS D, etc.; see Table 1). Of the 30 pink mutants, 21 were chosen for further study; the remainder were either leaky for growth on ADE D plates or contaminated with wild-type cells.

The Ade$^-$ mutants were then subjected to complementation analysis and phenotypic testing. To determine the number of loci defined by the mutants, all 21 mutants were mated to a single pink, Ade$^-$ tester strain (strain #2). Mating was carried out by mixing cell suspensions (OD_{600} = 1) and plating the mixtures in 10 µl aliquots on YEPD plates. The cells were then replicated to SPOR media (0.5% Na acetate, 1% KCl, 1% glucose, 1% agar) and incubated overnight at 30°C. The cells were then replica-plated to ADE D plates for scoring of phenotype. As shown in Table 3, some combinations of mutants failed to give Ade$^+$ colonies (possibly defining the same genetic locus as in strain #2), while others gave rise to numerous Ade$^+$ colonies (possibly defining a separate genetic locus). Because mutant #3 gave Ade$^+$ colonies when mated to #2, complementation testing was repeated with mutant #3. If the group of mutants defined two genetic loci, then all mutants that failed to give Ade$^+$ colonies when mated to strain #2 should give Ade$^+$ colonies when mated to #3. Results of the crosses are shown in Table 3.

Table 3

Mutant	x Mutant #2	x Mutant #3
#1	+	-
#3	+	-
#10	+	-
#15	+	-
#18	+	-
#24	+	-
#28	+	-
#30	+	-
#2	-	+
#6	-	+
#8	-	+
#9	-	+
#11	-	+
#17	-	+
#19	-	+
#20	-	+
#22	-	+
#27	-	+
#4	+	+
#12	+	+
#16	+	+

As shown in Table 3, most mutants fell into one of two groups, consistent with the idea that there are two adenine biosynthetic genes that, when missing, result in pink colonies on limiting adenine media. Three colonies (#4, #12, and #16) may either define a third locus or exhibit intragenic complementation. Two intensely pigmented mutants from each of the two complementation groups (#3 and #10; #6 and #11) were selected for further characterization. Additional analysis indicated that Ade^- was the only auxotrophy present in these strains.

A *P. methanolica* clone bank was constructed in the vector pRS426, a shuttle vector comprising 2μ and *S. cerevisiae URA3* sequences, allowing it to be propagated in *S. cerevisiae*. Genomic DNA was prepared from strain CBS6515 according to standard procedures. Briefly, cells were cultured overnight in rich media, spheroplasted with zymolyase, and lysed with SDS. DNA was precipitated from the lysate with ethanol and extracted with a phenol/chloroform mixture, then precipitated with ammonium acetate and ethanol. Gel electrophoresis of the DNA preparation showed the presence of intact, high molecular weight DNA and appreciable quantities of RNA. The DNA was partially digested with Sau 3A by incubating the DNA in the presence of a dilution series of the enzyme. Samples of the digests were analyzed by electrophoresis to determine the size distribution of fragments. DNA migrating between 4 and 12 kb was cut from the gel and extracted from the gel slice. The size-fractionated DNA was then ligated to pRS426 that had been digested with Bam HI and treated with alkaline phosphatase. Aliquots of the reaction mixture were electroporated in *E. coli* MC1061 cells using a BioRad Gene Pulser™ device as recommended by the manufacturer.

The genomic library was used to transform *S. cerevisiae* strain HBY21A (*ade2 ura3*) by electroporation (Becker and Guarente, Methods Enzymol. 194:182-187, 1991). The cells were resuspended in 1.2 M sorbitol, and six 300-μl aliquots were plated onto ADE D, ADE DS, URA D and URA DS plates (Table 1). Plates were incubated at 30°C for 4-5 days. No Ade^+ colonies were recovered on the ADE D or ADE DS plates. Colonies from the URA D and URA DS plates were replica-plated to ADE D plates, and two closely spaced, white colonies were obtained. These colonies were restreaked and confirmed to be Ura^+ and Ade^+. These two strains, designated Ade1 and Ade6, were streaked onto media containing 5 FOA (5 fluoro orotic acid; Sikorski and Boeke, Methods Enzymol. 194:302-318). Ura^- colonies were obtained, which were found to be Ade^- upon replica plating. These results indicate that the Ade^+ complementing activity is genetically linked to the plasmid-borne URA3 marker. Plasmids obtained from yeast strains Ade1 and Ade6 appeared to be identical by restriction mapping as described below. These genomic clones were designated pADE1-1 and pADE1-6, respectively.

Total DNA was isolated from the HBY21A transformants Ade1 and Ade6 and used to transform *E. coli* strain MC1061 to Amp^R. DNA was prepared from 2 Amp^R colonies of Ade1 and 3 Amp^R colonies of Ade6. The DNA was digested with Pst I, Sca I, and Pst I + Sca I and analyzed by gel electrophoresis. All five isolates produced the same restriction pattern.

PCR primers were designed from the published sequence of the *P. methanolica ADE2* gene (also known as *ADE1*; Hiep et al., Yeast 9:1251-1258, 1993). Primer 9080 (SEQ ID

NO:3) was designed to prime at bases 406-429 of the *ADE2* DNA (SEQ ID NO:1), and primer 9079 (SE ID NO:4) was designed to prime at bases 2852-2829. Both primers included tails to introduce Avr II and Spe I sites at each end of the amplified sequence. The predicted size of the resulting PCR fragment was 2450 bp.

PCR was carried out using plasmid DNA from the five putative *ADE2* clones as template DNA. The 100 µl reaction mixtures contained 1x Taq PCR buffer (Boehringer Mannheim, Indianapolis, IN), 10-100 ng of plasmid DNA, 0.25 mM dNTPs, 100 pmol of each primer, and 1 µl Taq polymerase (Boehringer Mannheim). PCR was run for 30 cycles of 30 seconds at 94°C, 60 seconds at 50°C, and 120 seconds at 72°C. Each of the five putative ADE2 genomic clones yielded a PCR product of the expected size (2.4 kb). Restriction mapping of the DNA fragment from one reaction gave the expected size fragments when digested with Bgl II or Sal I.

The positive PCR reactions were pooled and digested with Spe I. Vector pRS426 was digested with Spe I and treated with calf intestinal phosphatase. Four µl of PCR fragment and 1 µl of vector DNA were combined in a 10 µl reaction mix using conventional ligation conditions. The ligated DNA was analyzed by gel electrophoresis. Spe I digests were analyzed to identify plasmids carrying a subclone of the *ADE2* gene within pRS426. The correct plasmid was designated pCZR118.

Because the *ADE2* gene in pCZR118 had been amplified by PCR, it was possible that mutations that disabled the functional character of the gene could have been generated. To test for such mutations, subclones with the desired insert were transformed singly into *Saccharomyces cerevisiae* strain HBY21A. Cells were made electrocompetent and transformed according to standard procedures. Transformants were plated on URA D and ADE D plates. Three phenotypic groups were identified. Clones 1, 2, 11, and 12 gave robust growth of many transformants on ADE D. The transformation frequency was comparable to the frequency of Ura^+ transformants. Clones 6, 8, 10, and 14 also gave a high efficiency of transformation to both Ura^+ and Ade^+, but the Ade^+ colonies were somewhat smaller than those in the first group. Clone 3 gave many Ura^+ colonies, but no Ade^+ colonies, suggesting it carried a non-functional *ade2* mutation. Clones 1, 2, 11, and 12 were pooled.

To identify the *P. methanolica ade2* complementation group, two representative mutants from each complementation group (#3 and #10; #6 and #11), which were selected on the basis of deep red pigmentation when grown on limiting adenine, were transformed with the cloned ADE gene. Two hundred ml cultures of early log phase cells were harvested by centrifugation at 3000 x g for 3 minutes and resuspended in 20 ml of fresh KD buffer (50 mM potassium phosphate buffer, pH 7.5, containing 25 mM DTT). The cells were incubated in this buffer at 30°C for 15 minutes. The cells were then harvested and resuspended in 200 ml of ice-cold STM (270 mM sucrose, 10 mM Tris, pH 7.5, 1 mM $MgCl_2$). The cells were harvested and resuspended in 100 ml of ice-cold STM. The cells were again harvested and resuspended in 3-5 ml of ice-cold STM. 100-µl aliquouts of electrocompetent cells from each culture were then

mixed with Not I-digested pADE1-1 DNA. The cell/DNA mixture was placed in a 2 mm electroporation cuvette and subjected to a pulsed electric field of 5 kV/cm using a BioRad Gene Pulser™ set to 1000Ω resistance and capacitance of 25 μF. After being pulsed, the cells were diluted by addition of 1 ml YEPD and incubated at 30°C for one hour. The cells were then harvested by gentle centrifugation and resuspended in 400 μl minimal selective media lacking adenine (ADE D). The resuspended samples were split into 200-μl aliqouts and plated onto ADE D and ADE DS plates. Plates were incubated at 30°C for 4-5 days. Mutants #6 and #11 gave Ade^+ transformants. No Ade^+ transformants were observed when DNA was omitted, hence the two isolates appeared to define the *ade2* complementation group. The *ADE2* sequence is shown in SEQ ID NO:1.

Example 2

The *P. methanolica* clone bank disclosed in Example 1 was used as a source for cloning the Alcohol Utilization Gene (*AUG1*). The clone bank was stored as independent pools, each representing about 200-250 individual genomic clones. 0.1 μl of "miniprep" DNA from each pool was used as a template in a polymerase chain reaction with PCR primers (8784, SEQ ID NO:5; 8787, SEQ ID NO:6) that were designed from an alignment of conserved sequences in alcohol oxidase genes from *Hansenula polymorpha*, *Candida boidini*, and *Pichia pastoris*. The amplification reaction was run for 30 cycles of 94°C, 30 seconds; 50°C, 30 seconds; 72°C, 60 seconds; followed by a 7 minute incubation at 72°C. One pool (#5) gave a ~600 bp band. DNA sequencing of this PCR product revealed that it encoded an amino acid sequence with ~70% sequence identity with the *Pichia pastoris* alcohol oxidase encoded by the *AOX1* gene and about 85% sequence identity with the *Hansenula polymorpha* alcohol oxidase encoded by the *MOX1* gene. The sequence of the cloned *AUG1* gene is shown in SEQ ID NO:2.

Sub-pools of pool #5 were analyzed by PCR using the same primers used in the initial amplification. One positive sub-pool was further broken down to identify a positive colony. This positive colony was streaked on plates, and DNA was prepared from individual colonies. Three colonies gave identical patterns after digestion with Cla I.

Restriction mapping of the genomic clone and PCR product revealed that the *AUG1* gene lay on a 7.5 kb genomic insert and that sites within the PCR fragment could be uniquely identified within the genomic insert. Because the orientation of the gene within the PCR fragment was known, the latter information provided the approximate location and direction of transcription of the *AUG1* gene within the genomic insert. DNA sequencing within this region revealed a gene with very high sequence similarity at the amino acid level to other known alcohol oxidase genes.

Example 3

ade2 mutant *P. methanolica* cells are transformed by electroporation essentially as disclosed above with an expression vector comprising the *AUG1* promoter and terminator,

human GAD65 DNA (Karlsen et al., Proc. Natl. Acad. Sci. USA 88:8337-8341, 1991), and *ADE2* selectable marker. Colonies are patched to agar minimal methanol plates (10 to 100 colonies per 100-mm plate) containing 20 g/L Bacto™-agar (Difco), 6.7 g/L yeast nitrogen base without amino acids (Difco), 10 g/L methanol, and 0.4 μg/L biotin. The agar is overlayed with nitrocellulose, and the plates are inverted over lids containing 1 ml of 50% methanol in water and incubated for 3 to 5 days at 30°C. The membrane is then transferred to a filter soaked in 0.2 M NaOH, 0.1% SDS, 35 mM dithiothreitol to lyse the adhered cells. After 30 minutes, cell debris is rinsed from the filter with distilled water, and the filter is neutralized by rinsing it for 30 minutes in 0.1 M acetic acid.

The filters are then assayed for adhered protein. Unoccupied binding sites are blocked by rinsing in TTBS-NFM (20 mM Tris pH 7.4, 0.1% Tween 20, 160 mM NaCl, 5% powdered nonfat milk) for 30 minutes at room temperature. The filters are then transferred to a solution containing GAD6 monoclonal antibody (Chang and Gottlieb, J. Neurosci. 8:2123-2130, 1988), diluted 1:1000 in TTBS-NFM. The filters are incubated in the antibody solution with gentle agitation for at least one hour, then washed with TTBS (20 mM Tris pH 7.4, 0.1% Tween 20, 160 mM NaCl) two times for five minutes each. The filters are then incubated in goat anti-mouse antibody conjugated to horseradish peroxidase (1 μg/ml in TTBS-NFM) for at least one hour, then washed three times, 5 minutes per wash with TTBS. The filters are then exposed to commercially available chemiluminescence reagents (ECL™; Amersham Inc., Arlington Heights, IL). Light generated from positive patches is detected on X-ray film.

To more accurately detect the level of GAD_{65} expression, candidate clones are cultured in shake flask cultures. Colonies are grown for two days on minimal methanol plates at 30°C as disclosed above. The colonies are used to inoculate 20 ml of minimal methanol media (6.7 g/L yeast nitrogen base without amino acids, 10 g/L methanol, 0.4 μg/L biotin) at a cell density of 1 x 10^6 cells/ml. The cultures are grown for 1-2 days at 30°C with vigorous shaking. 0.2 ml of 50% methanol is added to each culture daily. Cells are harvested by centrifugation and suspended in ice-cold lysis buffer (20 mM Tris pH 8.0, 40 mM NaCl, 2 mM PMSF, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin) at 10 ml final volume per 1 g cell paste. 2.5 ml of the resulting suspension is added to 2.5 ml of 400-600 micron, ice-cold, acid-washed glass beads in a 15-ml vessel, and the mixture is vigorously agitated for one minute, then incubated on ice for 1 minute. The procedure is repeated until the cells have been agitated for a total of five minutes. Large debris and unbroken cells are removed by centrifugation at 1000 x g for 5 minutes. The clarified lysate is then decanted to a clean container. The cleared lysate is diluted in sample buffer (5% SDS, 8 M urea, 100 mM Tris pH 6.8, 10% glycerol, 2 mM EDTA, 0.01% bromphenol glue) and electrophoresed on a 4-20% acrylamide gradient gel (Novex, San Diego, CA). Proteins are blotted to nitrocellose and detected with GAD6 antibody as disclosed above.

Clones exhibiting the highest levels of methanol-induced expression of foreign protein in shake flask culture are more extensively analyzed under high cell density fermentation

conditions. Cells are first cultivated in 0.5 liter of YEPD broth at 30°C for 1 - 2 days with vigorous agitation, then used to inoculate a 5-liter fermentation apparatus (e.g., BioFlow III; New Brunswick Scientific Co., Inc., Edison, NJ). The fermentation vessel is first charged with mineral salts by the addition of 57.8 g $(NH_4)_2SO_4$, 68 g KH_2PO_4, 30.8 g $MgSO_4 \cdot 7H_2O$, 8.6 g $CaSO_4 \cdot 2H_2O$, 2.0 g NaCl, and 10 ml antifoam (PPG). H_2O is added to bring the volume to 2.5 L, and the solution is autoclaved 40 minutes. After cooling, 350 ml of 50% glucose, 250 ml 10 X trace elements (Table 4), 25 ml of 200 µg/ml biotin, and 250 ml cell inoculum are added.

Table 4

10 X trace elements:

$FeSO_4 \cdot 7H_2O$	100mM	27.8 g/L
$CuSO_4 \cdot 5H_2O$	2mM	0.5 g/L
$ZnCl_2$	8mM	1.09 g/L
$MnSO_4 \cdot H_2O$	8mM	1.35 g/L
$CoCl_2 \cdot 6H_2O$	2mM	0.48 g/L
$Na_2MoO_4 \cdot 2H_2O$	1mM	0.24 g/L
H_3BO_3	8mM	0.5 g/L
KI	0.5mm	0.08 g/L
biotin		5mg/L
thiamine		0.5 g/L

Add 1-2 mls H_2SO_4 per liter to bring compounds into solution.

The fermentation vessel is set to run at 28°C, pH 5.0, and >30% dissolved O_2. The cells will consume the initial charge of glucose, as indicated by a sharp demand for oxygen during glucose consumption followed by a decrease in oxygen consumption after glucose is exhausted. After exhaustion of the initial glucose charge, a glucose-methanol feed supplemented with NH_4^+ and trace elements is delivered into the vessel at 0.2% (w/v) glucose, 0.2% (w/v) methanol for 5 hours followed by 0.1% (w/v) glucose, 0.4% (w/v) methanol for 25 hours. A total of 550 grams of methanol is supplied through one port of the vessel as pure methanol using an initial delivery rate of 12.5 ml/hr and a final rate of 25 ml/hr. Glucose is supplied through a second port using a 700 ml solution containing 175 grams glucose, 250 ml 10X trace elements, and 99 g $(NH_4)_2SO_4$. Under these conditions the glucose and methanol are simultaneously utilized, with the induction of GAD_{65} expression upon commencement of the glucose-methanol feed. Cells from the fermentation vessel are analyzed for GAD_{65} expression as described above for shake flask cultures.

Cells are removed from the fermentation vessel at certain time intervals and subsequently analyzed. Little GAD_{65} expression is observed during growth on glucose. Exhaustion of glucose leads to low level expression of the GAD_{65} protein; expression is enhanced by the addition of MeOH during feeding of the fermentation culture. The addition of methanol has a clear stimulatory effect of the expresion of human GAD_{65} driven by the methanol-responsive *AUG1* promoter.

Example 4

Transformation conditions were investigated to determine the electric field conditions, DNA topology, and DNA concentration that were optimal for efficient transformation of *P. methanolica*. All experiments used *P. methanolica ade2* strain #11. Competent cells were prepared as previously described. Electroporation was carried out using a BioRad Gene Pulser™.

Three field parameters influence transformation efficiency by electroporation: capacitance, field strength, and pulse duration. Field strength is determined by the voltage of the electric pulse, while the pulse duration is determined by the resistance setting of the instrument. Within this set of experiments, a matrix of field strength settings at various resistances was examined. In all experiments, the highest capacitance setting (25 μF) of the instrument was used. 100 μl aliquots of electrocompetent cells were mixed on ice with 10 μl of DNA that contained approximately 1 μg of the *ADE2* plasmid pCZR133 that had been linearized with the restriction enzyme Not I. Cells and DNA were transferred to 2 mm electroporation cuvettes (BTX Corp., San Diego, CA) and electropulsed at field strengths of 0.5 kV (2.5 kV/cm), 0.75 kV (3.75 kV/cm), 1.0 kV (5.0 kV/cm), 1.25 kV (6.25 kV/cm), and 1.5 kV (7.5 kV/cm). These field strength conditions were examined at various pulse durations. Pulse duration was manipulated by varying the instrument setting resistances to 200 ohms, 600 ohms, or "infinite" ohms. Pulsed cells were suspended in YEPD and incubated at 30°C for one hour, harvested, resuspended, and plated. Three separate sets of experiments were conducted. In each set, electroporation conditions of 0.75 kV (3.75 kV/cm) at a resistance of "infinite" ohms was found to give a dramatically higher transformation efficiency than other conditions tested (see Fig. 1).

After the optimal pulse conditions were established, the influence of DNA topology on transformation efficiency was investigated. Electrocompetent cells were mixed with 1 μg of uncut, circular pCZR133 or with 1 μg of Not I-digested pCZR133. In three separate experiments, an average of roughly 25 transformants were recovered with circular DNA while linear DNA yielded an average of nearly 1 x 10^4 transformants. These data indicate that linear DNA transforms *P. methanolica* with much greater efficiency than circular DNA.

Finally, the relationship between DNA concentration and transformation efficiency was investigated. Aliquots of linear pCZR133 DNA (1 ng, 10 ng, 100 ng and 1 μg in 10 μl H_2O) were mixed with 100 μl electrocompetent cells, and electroporation was carried out at 3.75 kV/cm and "infinite" ohms. The number of transformants varied from about 10 (1 ng DNA) to 10^4 (1 μg DNA) and was found to be proportional to the DNA concentration.

Example 5

Integration of transforming DNA into the genome of *P. methanolica* was detected by comparison of DNA from wild-type cells and stable, white transformant colonies. Two classes of integrative transformants were identified. In the first, transforming DNA was found to have integrated into a homologous site. In the second class, transforming DNA was found to

have replaced the endogenous *AUG1* open reading frame. While not wishing to be bound by theory, this second transformant is believed to have arisen by a "transplacement recombination event" (Rothstein, Methods Enzymol. 194:281-301, 1991) whereby the transforming DNA replaces the endogenous DNA via a double recombination event.

P. methanolica ade2 strain #11 was transformed to Ade^+ with Asp I-digested pCZR140, a Bluescript® (Stratagene Cloning Systems, La Jolla, CA)-based vector containing the *P. methanolica ADE2* gene and a mutant of *AUG1* in which the entire open reading frame between the promoter and terminator regions has been deleted (Fig. 2). Genomic DNA was prepared from wild-type and transformant cells grown for two days on YEPD plates at 30°C. About 100-200 µl of cells was suspended in 1 ml H_2O, then centrifuged in a microcentrifuge for 30 seconds. The cell pellet was recovered and resuspended in 400 µl of SCE + DTT + zymolyase (1.2 M sorbitol, 10 mM Na citrate, 10 mM EDTA, 10 mM DTT, 1-2 mg/ml zymolyase 100T) and incubated at 37°C for 10-15 minutes. 400 µl of 1% SDS was added, and the solution was mixed until clear. 300 µl of 5 M potassium acetate, pH 8.9 was added, and the solution was mixed and centrifuged at top speed in a microcentrifuge for five minutes. 750 µl of the supernatant was transferred to a new tube and extracted with an equal volume of phenol/chloroform. 600 µl of the resulting supernatant was recovered, and DNA was precipitated by the addition of 2 volumes of ethanol and centrifugation for 15 minutes in the cold. The DNA pellet was resuspended in 50 ml TE (10 mM Tris pH 8, 1 mM EDTA) + 100 µg/ml RNAase for about 1 hour at 65°C. 10-µl DNA samples were digested with Eco RI (5 µl) in a 100 µl reaction volume at 37°C overnight. DNA was precipitated with ethanol, recovered by centrifugation, and resuspended in 7.5 µl TE + 2.5 µl 5X loading dye. The entire 10 ml volume was applied to one lane of a 0.7% agarose in 0.5 X TBE (10 X TBE is 108 g/L Tris base 7-9, 55 g/L boric acid, 8.3 g/L disodium EDTA) gel. The gel was run at 100 V in 0.5 X TBE containing ethidium bromide. The gel was photographed, and DNA was electrophoretically transferred to a positively derivatized nylon membrane (Nytran® N+, Schleicher & Schuell, Keene, NH) at 400 mA, 20 mV for 30 minutes. The membrane was then rinsed in 2 X SSC, blotted onto denaturation solution for five minutes, neutralized in 2 X SSC, then cross-linked damp in a UV crosslinker (Stratalinker®, Stratagene Cloning Systems) on automatic setting. The blot was hybridized to a PCR-generated *AUG1* promoter probe using a commercially available kit (ECL™ kit, Amersham Corp., Arlington Heights, IL). Results indicated that the transforming DNA altered the structure of the *AUG1* promoter DNA, consistant with a homologous integration event (Fig. 2).

In a second experiment, *P. methanolica ade 2* strain #11 was transformed to Ade^+ with Not I-digested pCZR137, a vector containing a human GAD65 cDNA between the *AUG1* promoter and terminator (Fig. 3). Genomic DNA was prepared as described above from wild-type cells and a stable, white, Ade^+ transformant and digested with Eco RI. The digested DNA was separated by electrophoresis and blotted to a membrane. The blot was probed with a PCR-generated probe corresponding to either the *AUG1* open reading frame or the *AUG1* promoter.

Results demonstrated that the *AUG1* open reading frame DNA was absent from the transformant strain, and that the *AUG1* promoter region had undergone a significant rearrangement. These results are consistent with a double recombination event (transplacement) between the transforming DNA and the host genome (Fig. 3).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

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(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 206-442-6678

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3077 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTGTGGGCGT TCCACACTCC TTGCTTTTCA TAATCTCTGT GTATTGTTTT ATTCGCATTT 240
TGATTCTCTT ATTACCAGTT ATGTAGAAAG ATCGGCAAAC AAAATATCAA CTTTTATCTT 300
GAACGCTGAC CCACGGTTTC AAATAACTAT CAGAACTCTA TAGCTATAGG GGAAGTTTAC 360
TGCTTGCTTA AAGCGGCTAA AAAGTGTTTG GCAAATTAAA AAAGCTGTGA CAAGTAGGAA 420
CTCCTGTAAA GGGCCGATTC GACTTCGAAA GAGCCTAAAA ACAGTGACTA TTGGTGACGG 480
AAAATTGCTA AAGGAGTACT AGGGCTGTAG TAATAAATAA TGGAACAGTG GTACAACAAT 540
AAAAGAATGA CGCTGTATGT CGTAGCCTGC ACGAGTAGCT CAGTGGTAGA GCAGCAGATT 600
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TTTGCGGGTA GGAAGCAAGG TCTAGTTTTC GTCGTTTCGG ATGGTTTACG AAAGTATCAG 720
CCATGAGTGT TTCCCTCTGG CTACCTAATA TATTTATTGA TCGGTCTCTC ATGTGAATGT 780
TTCTTTCCAA GTTCGGCTTT CAGCTCGTAA ATGTGCAAGA AATATTTGAC TCCAGCGACC 840
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TAACTGATAA GTCGAATCAA CATCTTTAAA TCCTTTAGTT AAGATCTCTG CAGCGGCCAG 960
TATTAACCAA TAGCATATTC ACAGGCATCA CATCGGAACA TTCAGAATGG ACTCGCAAAC 1020
TGTCGGGATT TTAGGTGGTG GCCAACTTGG TCGTATGATC GTTGAAGCTG CACACAGATT 1080
GAATATCAAA ACTGTGATTC TCGAAAATGG AGACCAGGCT CCAGCAAAGC AAATCAACGC 1140
TTTAGATGAC CATATTGACG GCTCATTCAA TGATCCAAAA GCAATTGCCG AATTGGCTGC 1200
CAAGTGTGAT GTTTTAACCG TTGAGATTGA ACATGTTGAC ACTGATGCGT TGGTTGAAGT 1260
TCAAAAGGCA ACTGGCATCA AAATCTTCCC ATCACCAGAA ACTATTTCAT TGATCAAAGA 1320
TAAATACTTG CAAAAAGAGC ATTTGATTAA GAATGGCATT GCTGTTGCCG AATCTTGTAG 1380

TGTTGAAAGT AGCGCAGCAT CTTTAGAAGA AGTTGGTGCC AAATACGGCT TCCCATACAT 1440

GCTAAAATCT AGAACAATGG CCTATGACGG AAGAGGTAAT TTTGTTGTCA AAGACAAGTC 1500

ATATATACCT GAAGCTTTGA AAGTTTTAGA TGACAGGCCG TTATACGCCG AGAAATGGGC 1560

TCCATTTTCA AAGGAGTTAG CTGTTATGGT TGTGAGATCA ATCGATGGCC AAGTTTATTC 1620

CTACCCAACT GTTGAAACCA TCCACCAAAA CAACATCTGT CACACTGTCT TTGCTCCAGC 1680

TAGAGTTAAC GATACTGTCC AAAAGAAGGC CCAAATTTTG GCTGACAACG CTGTCAAATC 1740

TTTCCCAGGT GCTGGTATCT TTGGTGTTGA AATGTTTTTA TTACAAAATG GTGACTTATT 1800

AGTCAACGAA ATTGCCCCAA GACCTCACAA TTCTGGTCAC TATACCATCG ACGCTTGTGT 1860

CACCTCGCAA TTTGAAGCTC ATGTTAGGGC CATTACTGGT CTACCCATGC CGAAGAACTT 1920

CACTTGTTTG TCGACTCCAT CTACCCAAGC TATTATGTTG AACGTTTTAG GTGGCGATGA 1980

GCAAAACGGT GAGTTCAAGA TGTGTAAAAG AGCACTAGAA ACTCCTCATG CTTCTGTTTA 2040

CTTATACGGT AAGACTACAA GACCAGGCAG AAAAATGGGT CACATTAATA TAGTTTCTCA 2100

ATCAATGACT GACTGTGAGC GTAGATTACA TTACATAGAA GGTACGACTA ACAGCATCCC 2160

TCTCGAAGAA CAGTACACTA CAGATTCCAT TCCGGGCACT TCAAGCAAGC CATTAGTCGG 2220

TGTCATCATG GGTTCCGATT CGGACCTACC AGTCATGTCT CTAGGTTGTA ATATATTGAA 2280

GCAATTTAAC GTTCCATTTG AAGTCACTAT CGTTTCCGCT CATAGAACCC CACAAAGAAT 2340

GGCCAAGTAT GCCATTGATG CTCCAAAGAG AGGGTTGAAG TGCATCATTG CTGGTGCTGG 2400

TGGTGCCGCT CATTTACCGG GAATGGTTGC GGCGATGACG CCGCTGCCTG TTATTGGTGT 2460

CCCTGTTAAA GGCTCTACTT TGGATGGTGT TGATTCACTA CACTCCATCG TTCAAATGCC 2520

AAGAGGTATT CCTGTTGCTA CTGTGGCTAT TAACAATGCT ACTAACGCTG CCTTGCTAGC 2580

TATCACAATC TTAGGTGCCG GCGATCCAAA TACTTGTCTG CAATGGAAGT TTATATGAAC 2640

AATATGGAAA ATGAAGTTTT GGGCAAGGCT GAAAAATTGG AAAATGGTGG ATATGAAGAA 2700

TACTTGAGTA CATACAAGAA GTAGAACCTT TTATATTTGA TATAGTACTT ACTCAAAGTC 2760

TTAATTGTTC TAACTGTTAA TTTCTGCTTT GCATTTCTGA AAAGTTTAAG ACAAGAAATC 2820

TTGAAATTTC TAGTTGCTCG TAAGAGGAAA CTTGCATTCA AATAACATTA ACAATAAATG 2880

ACAATAATAT ATTATTTCAA CACTGCTATA TGGTAGTTTT ATAGGTTTGG TTAGGATTTG 2940

AGATATTGCT AGCGCTTATC ATTATCCTTA ATTGTTCATC GACGCAAATC GACGCATTTC 3000

CACAAAAATT TTCCGAACCT GTTTTTCACT TCTCCAGATC TTGGTTTAGT ATAGCTTTTG 3060

ACACCTAATA CCTGCAG 3077

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3386 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATTCCTGC AGCCCGGGGG ATCGGGTAGT GGAATGCACG GTTATACCCA CTCCAAATAA 60
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CCCTAATCTG TTAACAGTCT CGGAGTATAC AAAAAAGTAA GTCAAATATC AAGGTGGCCG 180
GGGGCAGCAT CGAGACTCGA GATGGTACAT ACTTAAAAGC TGCCATATTG AGGAACTTCA 240
AAGTTTTATC TGTTTTTAGA ATTAAAAGAC GATTGTTGTA ACAAAACGTT GTGCCTACAT 300
AAACTCAAAT TAATGGAAAT AGCCTGTTTT GAAAAATACA CCTTCTTAAG TACTGACAAA 360
GTTTTGTTAA ATGACTATCG AACAAGCCAT GAAATAGCAC ATTTCTGCCA GTCACTTTTA 420
ACACTTTCCT GCTTGCTGGT TGACTCTCCT CATACAAACA CCCAAAAGGG AAACTTTCAG 480
TGTGGGGACA CTTGACATCT CACATGCACC CCAGATTAAT TTCCCCAGAC GATGCGGAGA 540
CAAGACAAAA CAACCCTTTG TCCTGCTCTT TTCTTTCTCA CACCGCGTGG GTGTGTGCGC 600
AGGCAGGCAG GCAGGCAGCG GGCTGCCTGC CATCTCTAAT CGCTGCTCCT CCCCCCTGGC 660
TTCAAATAAC AGCCTGCTGC TATCTGTGAC CAGATTGGGA CACCCCCCTC CCCTCCGAAT 720
GATCCATCAC CTTTTGTCGT ACTCCGACAA TGATCCTTCC CTGTCATCTT CTGGCAATCA 780
GCTCCTTCAA TAATTAAATC AAATAAGCAT AAATAGTAAA ATCGCATACA AACGTCATGA 840
AAAGTTTTAT CTCTATGGCC AACGGATAGT CTATCTGCTT AATTCCATCC ACTTTGGGAA 900
CCGCTCTCTC TTTACCCCAG ATTCTCAAAG CTAATATCTG CCCCTTGTCT ATTGTCCTTT 960
CTCCGTGTAC AAGCGGAGCT TTTGCCTCCC ATCCTCTTGC TTTGTTTCGG TTATTTTTTT 1020
TTCTTTTGAA ACTCTTGGTC AAATCAAATC AAACAAAACC AAACCTTCTA TTCCATCAGA 1080
TCAACCTTGT TCAACATTCT ATAAATCGAT ATAAATATAA CCTTATCCCT CCCTTGTTTT 1140
TTACCAATTA ATCAATCTTC AAATTTCAAA TATTTTCTAC TTGCTTTATT ACTCAGTATT 1200

AACATTTGTT TAAACCAACT ATAACTTTTA ACTGGCTTTA GAAGTTTTAT TTAACATCAG 1260
TTTCAATTTA CATCTTTATT TATTAACGAA ATCTTTACGA ATTAACTCAA TCAAAACTTT 1320
TACGAAAAAA AAATCTTACT ATTAATTTCT CAAAATGGCT ATTCCAGATG AATTTGATAT 1380
TATTGTTGTC GGTGGTGGTT CCACCGGTTG TGCTCTTGCT GGTAGATTAG GTAACTTGGA 1440
CGAAAACGTC ACAGTTGCTT TAATCGAAGG TGGTGAAAAC AACATCAACA ACCCATGGGT 1500
TTACTTACCA GGTGTTTATC CAAGAAACAT GAGATTAGAC TCAAAGACTG CTACTTTTTA 1560
CTCTTCAAGA CCATCACCAC ACTTGAACGG TAGAAGAGCT ATTGTTCCAT GTGCTAACAT 1620
CTTGGGTGGT GGTTCTTCCA TCAACTTCTT GATGTACACC AGAGCCTCTG CCTCCGATTA 1680
CGATGATTGG GAATCTGAAG GTTGGACTAC CGATGAATTA TTACCACTAA TGAAGAAGAT 1740
TGAAACTTAT CAAAGACCAT GTAACAACAG AGAATTGCAC GGTTTCGATG GTCCAATTAA 1800
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TCAAGGTATT CCATTTGTTG ATGATGCTGA AGATTTGAAA TGTTCCCACG GTGCTGAGCA 1920
CTGGTTGAAG TGGATCAACA GAGACTTAGG TAGAAGATCC GATTCTGCTC ATGCTTACAT 1980
TCACCCAACC ATGAGAAACA AGCAAAACTT GTTCTTGATT ACTTCCACCA AGTGTGAAAA 2040
GATTATCATT GAAAACGGTG TTGCTACTGG TGTTAAGACT GTTCCAATGA AGCCAACTGG 2100
TTCTCCAAAG ACCCAAGTTG CTAGAACTTT CAAGGCTAGA AAGCAAATTA TTGTTTCTTG 2160
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TCACTACTGT TTCTTCACTC CATACCATGT CAAGCCAGAT ACTCCATCAT TCGATGACTT 2340
TGTTAGAGGT GATAAAGCTG TTCAAAAATC TGCTTTCGAC CAATGGTATG CTAACAAGGA 2400
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AAGAATGGAC TGTTTTGCCG GTGAAGTTAC TTCTCACCAC CCACACTACC CATACGACTC 2820
ACCAGCCAGA GCTGCTGACA TGGACTTGGA AACTACTAAA GCTTATGCTG GTCCAGACCA 2880
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TTGCCCAGAT AATGTTGGTT GTAACACTTA CTCTACTGCT TTGTTAATCG GTGAAAAGGC 3240

TTCTACCTTA GTTGCTGAAG ACTTGGGCTA CTCTGGTGAT GCTTTGAAGA TGACTGTTCC 3300

AAACTTCAAA TTGGGTACTT ATGAAGAAGC TGGTCTAGCT AGATTCTAGG GCTGCCTGTT 3360

TGGATATTTT TATAATTTTT GAGAGT 3386

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGATCACCTA GGACTAGTGA CAAGTAGGAA CTCCTGTA 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAGCTGCCTA GGACTAGTTT CCTCTTACGA GCAACTAGA 39

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGGTTGAAGT GGATCAA 17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTGTGGTCAC CGAAGAA 17

Claims

What is claimed is:

1. A method for introducing a DNA molecule into a *Pichia methanolica* cell, comprising exposing a *Pichia methanolica* cell, in the presence of a linear DNA molecule, to an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm and a time constant of from 1 to 40 milliseconds, whereby said DNA molecule is introduced into said cell.

2. A method according to claim 1 wherein said DNA molecule comprises a segment encoding a polypeptide, other than a *P. methanolica* polypeptide, operably linked to a *P. methanolica* gene transcription promoter and a *P. methanolica* gene transcription terminator.

3. A method according to claim 2 wherein said transcription promoter is a *P. methanolica AUG1* gene promoter.

4. A method according to claim 3 wherein said *AUG1* gene promoter comprises a sequence of nucleotides as shown in SEQ ID NO:2 from nucleotide 24 to nucleotide 1354.

5. A method according to claim 2 wherein said DNA molecule further comprises a selectable marker gene that complements a mutation in said cell.

6. A method according to claim 5 wherein said selectable marker gene is a *P. methanolica* gene.

7. A method according to claim 5 wherein said marker gene is a *P. methanolica ADE2* gene.

8. A method according to claim 7 wherein said *ADE2* gene comprises a sequence of nucleotides as shown in SEQ ID NO:1 from nucleotide 407 to nucleotide 2851.

9. A method according to claim 1 wherein said DNA molecule comprises a selectable marker gene that complements a mutation in said cell.

10. A method according to claim 9 wherein said marker gene is a *P. methanolica* gene.

11. A method according to claim 9 wherein said marker gene is a *P. methanolica ADE2* gene.

12. A method according to claim 11 wherein said *ADE2* gene comprises a sequence of nucleotides as shown in SEQ ID NO:1 from nucleotide 407 to nucleotide 2851.

13. A method according to claim 1 wherein said time constant is from 10 to 30 milliseconds.

14. A method for transforming *P. methanolica* with heterologous DNA, comprising exposing a population of *P. methanolica* cells, in the presence of heterologous linear DNA molecules, to an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm and a time constant of from 1 to 40 milliseconds, whereby said heterologous DNA is introduced into at least a portion of said population, and recovering cells into which said DNA has been introduced.

15. A method according to claim 14 wherein from 10^3 to 10^5 cells are recovered per microgram of heterologous DNA.

16. A method according to claim 15 wherein from 0.9×10^4 to 1.1×10^4 cells are recovered per microgram of heterologous DNA.

17. A method according to claim 14 further comprising the step of recovering integrative transformants from said recovered cells.

18. A method according to claim 17 wherein said recovering step comprises culturing said cells in a growth medium comprising sorbitol as a carbon source.

19. A method according to claim 14 wherein said population of cells is in early log phase growth.

20. A method according to claim 14 wherein said time constant is from 10 to 30 milliseconds.

21. A *Pichia methanolica* cell transformed by the method of claim 1.

22. A *Pichia methanolica* cell transformed by the method of claim 6.

TRANSFORMATION OF *PICHIA METHANOLICA*

Fig. 1

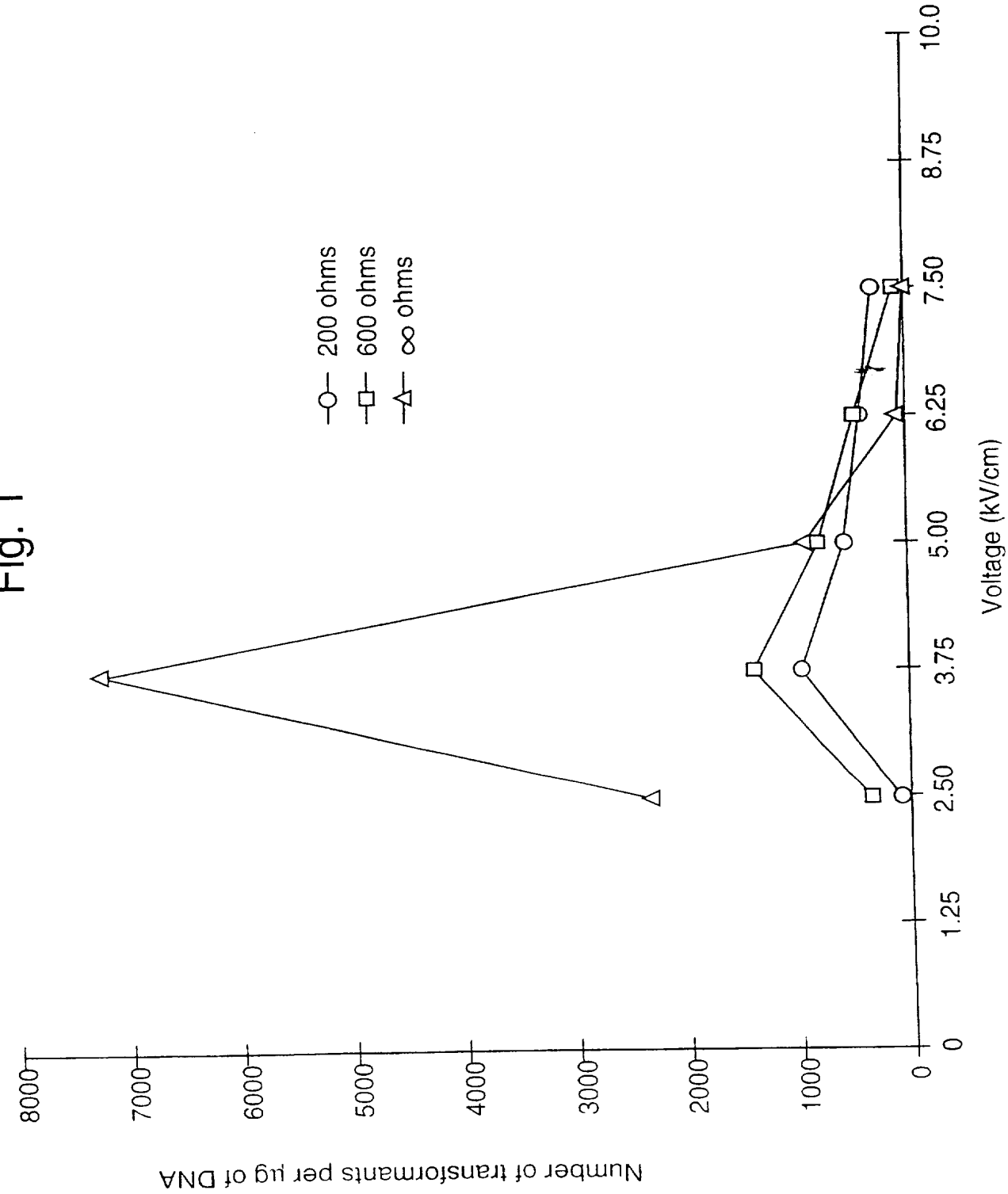

200 ohms
600 ohms
∞ ohms
Number of transformants per µg of DNA
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1.25
2.50
3.75
5.00
6.25
7.50
8.75
10.0
Voltage (kV/cm)

Fig. 2

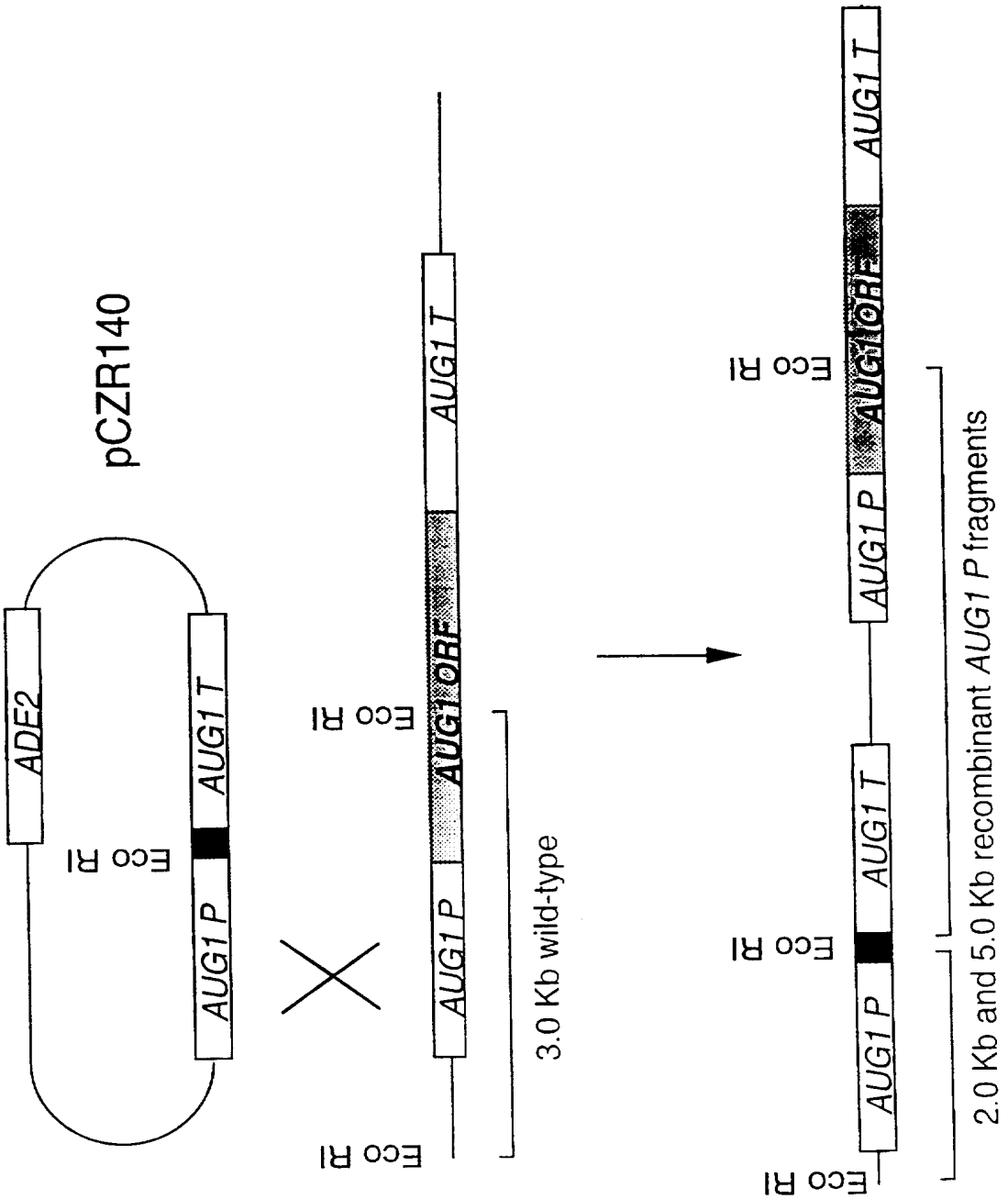
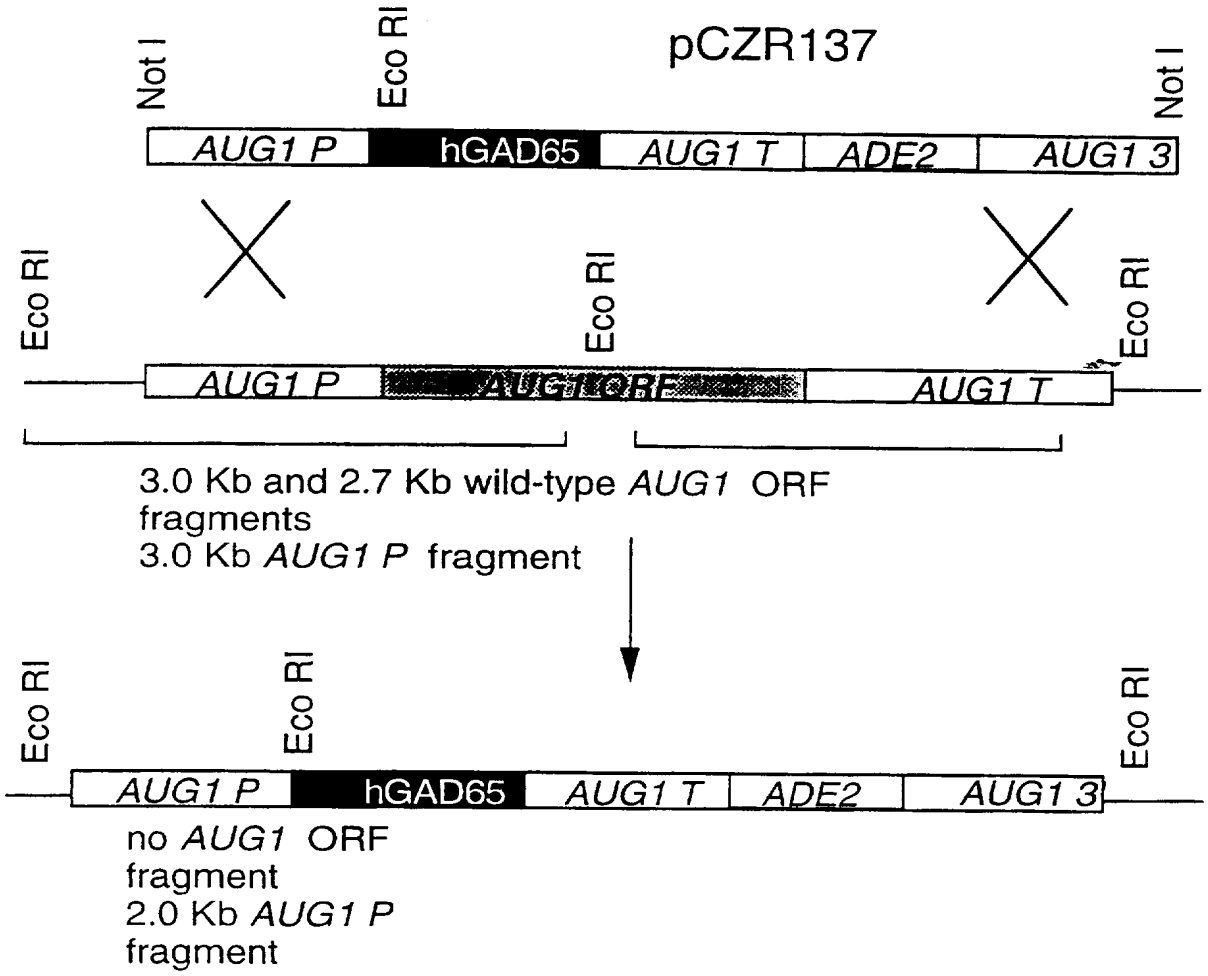

pCZR140
ADE2
AUG1 T
Eco RI
AUG1 P
Eco RI
AUG1 T
AUG1 ORF
AUG1 P
Eco RI
3.0 Kb wild-type
Eco RI
AUG1 T
AUG1 ORF
AUG1 P
Eco RI
AUG1 P
AUG1 T
Eco RI
2.0 Kb and 5.0 Kb recombinant AUG1 P fragments

Fig. 3


pCZR137
Not I
Eco RI
Not I
AUG1 P
hGAD65
AUG1 T
ADE2
AUG1 3
Eco RI
Eco RI
Eco RI
AUG1 P
AUG1 ORF
AUG1 T
3.0 Kb and 2.7 Kb wild-type AUG1 ORF fragments
3.0 Kb AUG1 P fragment
Eco RI
Eco RI
Eco RI
AUG1 P
hGAD65
AUG1 T
ADE2
AUG1 3
no AUG1 ORF fragment
2.0 Kb AUG1 P fragment

INTERNATIONAL SEARCH REPORT

Interr ·nal Application No
PCT/US 97/12581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N15/81 C12N1/19 C12N15/90 //(C12N1/19, C12R1:84)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HIEP T T ET AL: "Transformation in the methylotrophic yeast Pichia methanolica utilizing homologous ADE1 and heterologous Saccharomyces cerevisiae ADE2 and LEU2 genes as genetic markers." YEAST, (1993 NOV) 9 (11) 1189-97. JOURNAL CODE: YEA. ISSN: 0749-503X., XP000653248 see abstract see page 1189, paragraph 2 - page 1190, paragraph 1; figure 1 --- -/--	1,2,5-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 1997	29/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/12581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MEILHOC, E. ET AL.: "High efficiency transformation of intact yeast cells by electric field pulses" BIO/TECHNOLOGY, vol. 8, March 1990, pages 223-227, XP002049051 cited in the application see abstract see page 223, paragraph 5 see page 224, paragraph 6; figure 1 see page 225, paragraph 1; figure 2 see page 226, paragraph 2; figure 3 ---	1,2, 5-17, 19-22
Y	US 4 857 467 A (SREEKRISHNA KOTIKANYADAN ET AL) 15 August 1989 see column 3, line 21 - line 32 see column 6, line 6 - line 22 see column 9, line 2 - line 9; table I ---	18
P,X	WO 97 17450 A (ZYMOGENETICS INC) 15 May 1997 see abstract; claims 1-4,6-8,11,13-17,19-22; figures 1-3 see page 11, line 16 - page 13, line 7; claims 25,34-36,40-45 ---	1-22
A	HIEP T T ET AL: "THE 5-AMINOIMIDAZOLE RIBONUCLEOTIDE-CARBOXYLASE STRUCTURAL GENE OF THE METHYLOTROPHIC YEAST PICHIA METHANOLICA: CLONING, SEQUENCING AND HOMOLOGY ANALYSIS" YEAST, vol. 9, 1993, pages 1251-1258, XP000653249 see abstract; figures 2,3 -----	8,12

2

INTERNATIONAL SEARCH REPORT

.formation on patent family members

Inter ›nal Application No
PCT/US 97/12581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4857467 A	15-08-89	NONE	
WO 9717450 A	15-05-97	AU 1158297 A	29-05-97
		AU 7673796 A	29-05-97
		WO 9717451 A	15-05-97